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Geographical distribution of the cryptic species *Agrodiaetus alcestis alcestis, A. alcestis karacetinae* and *A. demavendi* (Lepidoptera: Lycaenidae) revealed by cytogenetic analysis

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Abstract. *Agrodiaetus alcestis* (Zerny, 1932) and *A. demavendi* (Pfeiffer, 1938) belong to the "brown" complex of the genus *Agrodiaetus* Hübner, 1822. This complex includes several cryptic species which are extremely uniform in wing colouration and genitalia structure, but have distinct chromosome numbers. In this paper we analyse karyotypes of *A. alcestis karacetinae* Lukhtanov et Dantchenko, 2002 and *A. demavendi* in populations from Iran. We demonstrate that *A. alcestis karacetinae* and *A. demavendi* are sympatric in the provinces Esfahan, Lorestan, Hamadan, Kurdestan, Kermanshah, and Markazi. The haploid chromosome number of *A. alcestis karacetinae* is found to be n=19 in all the populations studied. The karyotype of *A. demavendi* is not stable. The lowest chromosome numbers n=63-67 is observed in the south of the revealed distribution range (provinces Esfahan and Lorestan). The highest chromosome numbers (n=73-74) is found in Northwestern Iran in provinces Kurdestan and Zanjan. We also confirm that *A. alcestis* sensu lato appears as a polyphyletic taxon on the Bayesian phylogenetic tree inferred from the mitochondrial *COI* barcodes and should be most likely divided in two different species: *A. alcestis* sensu stricto and *A. karacetinae*. The new data on occurrence of *A. admetus* and *A. ripartii* in Iran are discussed.

Key words: *Agrodiaetus*, butterfly, chromosome, *COI*, DNA barcoding, cryptic species, Iran, karyotype, Lepidoptera, Lycaenidae.

Introduction

Agrodiaetus alcestis Zerny, 1932 and *A. demavendi* (Pfeiffer, 1938) are members of so called "brown" complex of the genus *Agrodiaetus* Hübner, 1822 (Lepidoptera: Lycaenidae) and distributed in Southwest Asia. This complex consists of two groups of species recognized as sister clades in all published phylogenetic reconstructions (Wiemers, 2003; Kandul et al., 2004, 2007; Lukhtanov et al., 2005): the *A. dolus* (Hübner, [1823]) – *A. alcestis* group and *A. admetus* (Esper, [1783]) – *A. demavendi* group. These clades comprise numerous monomorphic species in which both females and males have similar brown coloration of the upperside of the wings (Lukhtanov et al., 2003). The species are also similar in wing colour pattern and genitalia

structure. In contrast to morphological uniformity, the complex possesses a great chromosome number diversity, and each species has a specific karyotype (de Lesse, 1960a, 1960b; Lukhtanov et al., 1998; Lukhtanov, Dantchenko, 2002a, b; Lukhtanov et al., 2005; Kandul et al., 2007).

De Lesse (1960a, 1960b), who first studied this complex karyologically, showed that species description, species determination and study of species distribution ranges are impossible without karyotype investigation. De Lesse (1960b) mapped distribution of several "brown" species from north and northwest Iran and Turkey. He ascertained that *A. alcestis* and *A. demavendi* had variable chromosome numbers (n=19-22 and n=67-74 correspondingly). Further studies (Larsen, 1975; Lukhtanov et al., 1998) showed that populations of *A. alcestis* can be divided in two groups with different chromosome numbers: western group with n=20-21 (populations of Lebanon and Turkey, except for SE Turkey) and oriental group with n=19 (Iranian populations, SE Turkey). Wiemers (2003; Wiemers et. al., 2009) established that *A. alcestis karacetinae* Lukhtanov et Dantchenko, 2002 with n=19 and *A. alcestis alcestis* with n=20-21 have similar nuclear ITS2 sequences but different and most likely independently evolved COI haplotypes indicating possible specific distinctness of these two taxa.

A. demavendi was shown to have a wide distribution range in Turkey, Iran, Armenia, and Azerbaijan (Lukhtanov et al., 1998) and to consist of several chromosomal races (Kandul et al., 2004; Lukhtanov et al., 2005; Wiemers et al., 2009).

In this study we analyzed karyotypes of *A. alcestis karacetinae* and *A. demavendi* from different localities of Western and Central Iran in order to reveal the southernmost and the easternmost limits of distribution ranges of these species. We also tested the Wiemers's hypothesis (Wiemers, 2003; Wiemers et al., 2009) about the polyphyly of *A. alcestis* sensu lato by using molecular phylogenetic methods.

Material and methods

Insects

Population samples of different taxa of the genus *Agrodiaetus* were collected by V. Lukhtanov, A. Dantchenko and N. Shapoval in Iran in the period of 2002-2009. In most cases GPS localities data were fixed (Table 1).

When collecting in the field, we used a protocol that allowed us to obtain molecular and chromosomal information from the same individual specimen (Bulatova et al., 2009). Fresh (not worn) adult males were used to investigate the karyotypes. After capturing a butterfly in the field, it was placed in a glassine envelope for 1-2 hours to keep it alive until we processed it. Testes were removed from the abdomen and placed into a small 0.5 ml vial with a freshly prepared fixative (ethanol and glacial acetic acid, 3:1). Then each wing was carefully removed from the body using two sets of forceps: (i) a coarse or "flattened" set to hold the body and (ii) a much finer set to pinch off the wings. The wingless body was placed into a plastic, 2 ml vial with pure 100% ethanol. Each vial with ethanol has already been numbered. This ID number was also used to label a vial with the fixative and a glassine envelope in which the wings are preserved. Thus, each specimen was individually fixed. After the fixation we had three components collected for each butterfly, each of which was identified by a common ID number: (a) a vial containing the butterfly testes (for karyotype analysis), (b) a vial containing the butterfly wingless body (for DNA analysis) and (c)

Table 1. List of the studied *Agrodiaetus* samples with their haploid numbers (n) and locality data.

Taxon	ID number	n	Province	Locality	Altitude	Collected by (year)
Agrodiaetus admetus	E456	80	Zanjan (West part)	10 km W Dandy	1900-2000 m	V.Lukhtanov, A.Dantchenko (2004)
	E493	77	Azerbaijan-e-Gharbi	Takab, 10 km E Takht-e-Suleyman, to the S from the road	2250 m	V.Lukhtanov, A.Dantchenko (2004)
	M761	77	Ardebil	Khalkhal, Gollijeh	1900 m	V.Lukhtanov, A.Dantchenko (2005)
Agrodiaetus alcestis karacetinae	N504; N512	19	Qazvin	Avaj-Pass, 35°34' N/ 49°09' E	2200 m	V.Lukhtanov (2002)
	N538	19	Hamadan	Shah Pass, 34°5' N/ 48°11' E	2250 m	V.Lukhtanov (2002)
	F669; F672; F703	19	Markazi	SW 33°50' N/ 49°02' E	2500 m	V.Lukhtanov (2003)
	E439; E444	19	Zanjan (West part)	10 km W Dandy, 36°35' N/ 47°30' E	1900-2000 m	V.Lukhtanov, A.Dantchenko (2004)
	E407	19	Kurdestan	40 km SW Saqqez, 36°05' N/ 45°59' E	1800-1900 m	V.Lukhtanov, N. Shapoval (2007)
	Z514	19	-	between Kermanshah and Senandaj, Gerdene Morvari 34° 54.011' N/ 046° 56.436' E	1725 m	V.Lukhtanov, N. Shapoval (2007)
	Z643; Z644	19	-	14 km N of Chenareh 35° 41.269' N/ 46° 21.653' E	1855 m	V.Lukhtanov, N. Shapoval (2007)
	Z766; Z767	19	-	40 km SW Saqqez 36° 04.824' N/ 045° 58.883' E	1880 m	V.Lukhtanov, N. Shapoval (2007)
	Z850	19	-	Divandarreh 36° 08.541' N/ 046° 47.218' E	2130 m	V.Lukhtanov, N. Shapoval (2007)
	W164	19	-	W of Sanandaj 35° 25.244 N/ 46° 51.3324 E	2058 m	V.Lukhtanov, N. Shapoval (2009)
	W041	19	Esfahan	Fereydun-Shahr, 32° 57` N/ 50°03` E	2800 m	V.Lukhtanov, N. Shapoval (2009)
	W062; W067; W076; W108	19	Lorestan	Sarvand, 33°22.388 N/ 49°10.247 E	2070 m	V.Lukhtanov, N. Shapoval (2009)
Agrodiaetus demavendi	W042	ca. 62	Esfahan	Fereydun-Shahr, 32° 57` N/ 50°03` E	2800 m	V.Lukhtanov, N. Shapoval (2009)
	W058; W060	64	-	33°00.106 N/ 49°59.610 E	2800 m	V.Lukhtanov, N. Shapoval (2009)
	W070	63	Lorestan	Sarvand, 33°22.388 N/ 49°10.247 E	2070 m	V.Lukhtanov, N. Shapoval (2009)

Table 1. (Continuation).

Taxon	ID number	n	Province	Locality	Altitude	Collected by (year)
Agrodiaetus demavendi	W128	69	Kurdestan	Qorvah, 35°05.499 N/ 47°44.230 E	2238 m	V.Lukhtanov, N. Shapoval (2009)
	W130	ca. 74	-	Qorveh, 35°05.499 N/ 47°44.230 E	2238 m	V.Lukhtanov, N. Shapoval (2009)
	W162	ca. 64-69	-	W of Sanandaj 35°25.244 N/ 46°51.3324 E	2058 m	V.Lukhtanov, N. Shapoval (2009)
	W184	74	-	14 km N of Chenareh 35° 41.160' N/ 46° 21.293' E	1862 m	V.Lukhtanov, N. Shapoval (2009)
	E452	74	Zanjan (West part)	10 km W Dandy	1900-2000 m	V.Lukhtanov, A.Dantchenko (2004)
Agrodiaetus ripartii	N038	ca. 89	Azerbaijan-e-Sharqi	Ahar Pass, 20 km SW Ahar	1800-1850 m	V.Lukhtanov, A.Dantchenko (2005)

a glassine envelope containing the wings. The set specimens of the donor butterflies (the butterfly wingless bodies in ethanol and wings in glassine envelopes) are kept in the department of Karyosystematics, Zoological Institute of Russian Academy of Science.

Chromosome preparation and karyotyping

Testes were stored in the fixative for 1-12 months at +4°C. Then the gonads were stained in 2% acetic orcein for 30-60 days at +18-20°C. Different stages of male meiosis were examined by using a light microscope Jenaval, Carl Zeiss and photographed by Nikon Coolpix 4500. We have used an original two-phase method of chromosome analysis (Lukhtanov, Dantchenko, 2002a; Lukhtanov et al., 2006, 2008).

Sequence analysis and phylogeny inference

For molecular phylogenetical analysis we used *COI* barcodes (658-bp 5' segments of mitochondrial cytochrome oxidase subunit I) from 2 specimens of *A. alcestis alcestis*, 4 specimens of *A. alcestis karacetinae* and 30 other representatives of the *A. alcestis-A. dolus* clade. This fragment was selected as it was available from Genbank for almost all taxa of the "brown" complex, and its effectiveness for solving species-level taxonomical problems in butterflies was previously demonstrated (Wiemers, 2003; Hebert et al., 2004; Lukhtanov et al., 2009).

The *A. admetus - A. demavendi* clade was earlier inferred as a sister group to the *A. dolus - A. alcestis* clade (Kandul et al., 2004, 2007). Therefore we used as outgroups the representatives of the *A. admetus - A. demavendi* clade as well as A. *stempfferi* (Brandt, 1938), a phylogenetically distant species. All the sequences were found in GenBank (Wiemers, 2003; Kandul et al., 2004, 2007; Lukhtanov et al., 2005). The sequences were edited and aligned by ClustalW algorithm in BioEdit 7.0.3 software (Hall, 1999).

Neighbour-joining (NJ) analysis was performed using Kimura's two-parameter

model of base substitution as implemented in MEGA4 (Tamura et al., 2007). All positions containing missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option).

Maximum parsimony (MP) analysis was performed using a heuristic search as implemented in MEGA4 (Tamura et al., 2007). A heuristic search was carried out using the close-neighbour-interchange algorithm with search level 3 (Nei, Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). We used nonparametric bootstrap values (Felsenstein, 1985) to estimate branch support on the recovered tree. The bootstrap consensus trees were inferred from 1000 replicates by MEGA4 software for both NJ and MP analyses.

Bayesian analyses were performed using the program MrBayes 3.1.2 (Huelsenbeck, Ronquist, 2001; Ronquist, Huelsenbeck, 2003). A GTR substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites was specified before running the program for 5,000,000 generations with default settings. The first 1250 trees (out of 5000) were discarded as a burn-in prior to computing a consensus phylogeny and posterior probabilities.

Abbreviations:

ca. (circa) - approximately.

MI – meiotic metaphase I,

MII – meiotic metaphase II.

VL – sequence produced by Vladimir Lukhtanov with co-authors.

MW - sequence produced by Martin Wiemers.

Results

Karyotypes

A. alcestis karacetinae (Fig. 1, a)

The haploid chromosome number n=19 was found in MI and MII cells of twenty one studied individuals. In MI cells, all bivalents formed a gradient size row. The karyotype contained no exceptionally large or small bivalents.

A. demavendi (Fig.1, b)

In most cases the chromosome numbers were only approximately established. They are similar in several examined populations (Table 1). The karyotype contains 2 large and 2 medium-sized bivalents. All other bivalents are relatively small and form a gradient series in MI.

A. admetus (Fig. 1, c)

The haploid chromosome number n=77 was found in MI cells of two studied individuals. In the specimen E456 the number n=80 was found. In MI cells, the karyotype contains one large and three medium-sized bivalents. All other bivalents are relatively small and form a gradient series in MI cells.

A. ripartii Freyer, 1830 (Fig. 1, d)

The haploid chromosome number n=ca. 89 was found in MI cell of the single studied specimen. The count was done with approximation due to the overlapping of some chromosomes. In MI cells, the karyotype contains one large and one medium-sized bivalents. All other bivalents are relatively small and form a gradient series in MI cells.

Phylogenetic analysis of molecular data

We have analyzed 43 (including outgroup) *COI* barcode sequences. The final data set alignment included 690 sites, 106 sites were variable, and 71 sites were parsimony-informative. The average nucleotide frequencies were 0.329 (A), 0.367 (T), 0.155 (C), and 0.148 (G). The test of the homogeneity of substitution patterns between sequences did not reject the null hypothesis that the sequences have evolved with the

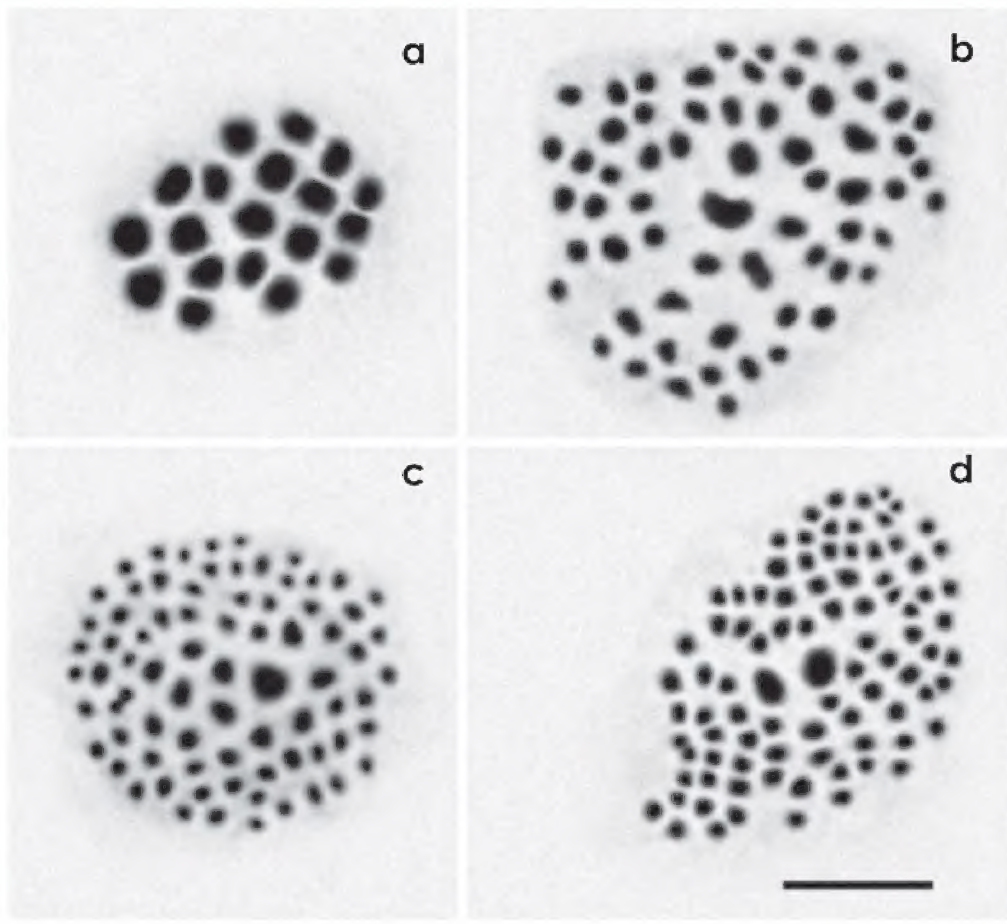

Fig. 1, a-d. *Agrodiaetus* karyotypes. **a** - *A. alcestis karacetinae* Lukhtanov et Dantchenko, 2003. ID W164, MI, n=19, Iran, Prov. Kurdestan. **b** -. *A. demavendi* (Pfeiffer, 1938). ID W070, MI, n=63, Iran, Prov. Lorestan. **c** - *A. admetus* (Esper, [1783]) . ID E493, MI, n=77, Iran, Prov. Azerbaijan-e-Gharbi. **d** - *A. ripartii* (Freyer, 1830). ID N038, MI, n=ca. 89, Iran, Prov. Azerbaijan-e-Sharqi. Scale bar = 10 μm.

same pattern of substitution. The disparity index indicated no larger differences in base composition biases than expected based on evolutionary divergence between the sequences and by chance alone. The NJ and MP bootstrap consensus trees are shown on the Fig. 2 and Fig. 3 correspondingly. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown above the branches. The 50% majority rule consensus tree was recovered from the trees sampled during Bayesian analyses and is shown on the Fig. 4. The posterior probability is shown above every branch on the Bayesian tree.

The Bayesian and NJ phylogenetic analyses support monophyly of *A. alcestis karacetinae* with n=19, however statistical support for this clade was relatively low. On the Bayesian tree *A. alcestis karacetinae* appeared as a taxon closely related to *A. dantchenkoi* Lukhtanov et Wiemers, 2003, not to *A. alcestis alcestis* as expected (Fig. 4). *A. alcestis alcestis* with n=21, 21 did not appear as monophyletic group on the MP and Bayesian trees; it appeared as monophyletic group only on the NJ tree but with low bootstrap support. The phylogenetic relationships between *A. alcestis karacetinae* and *A. alcestis alcestis* were not resolved on the NJ and MP trees. At the same time, on the Bayesian tree *A. alcestis* sensu lato (*A. alcestis karacetinae* + *A. alcestis alcestis*) appeared as a clearly polyphyletic taxon (Fig. 4).

Discussion

We found that in Esfahan, Lorestan, Hamadan, Kurdestan, Kermanshah, and

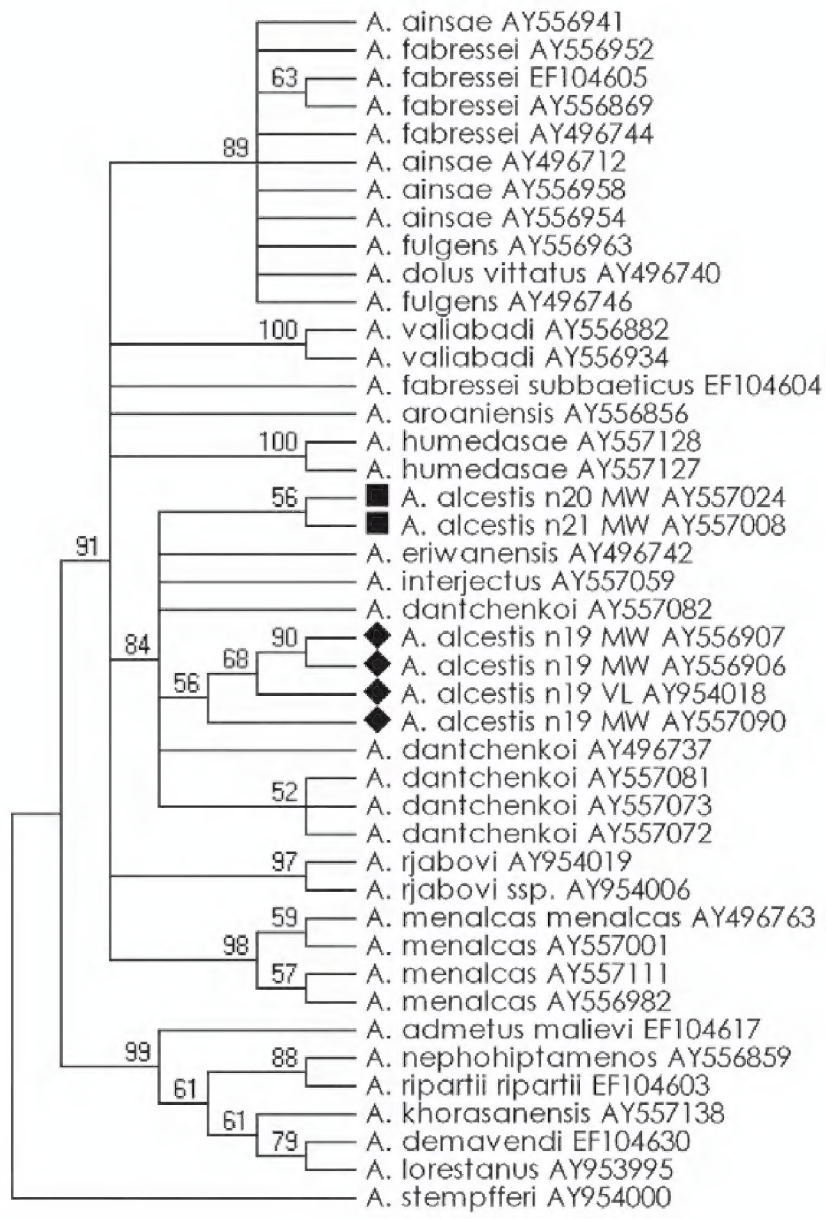

Fig. 2. Bootsrtap consensus NJ tree of the "brown" *Agrodiaetus* complex inferred from *COI* barcodes. Bootstrap values >50% are shown above the branches. Haploid chromosome number of *A. alcestis* are shown after name of a taxon.

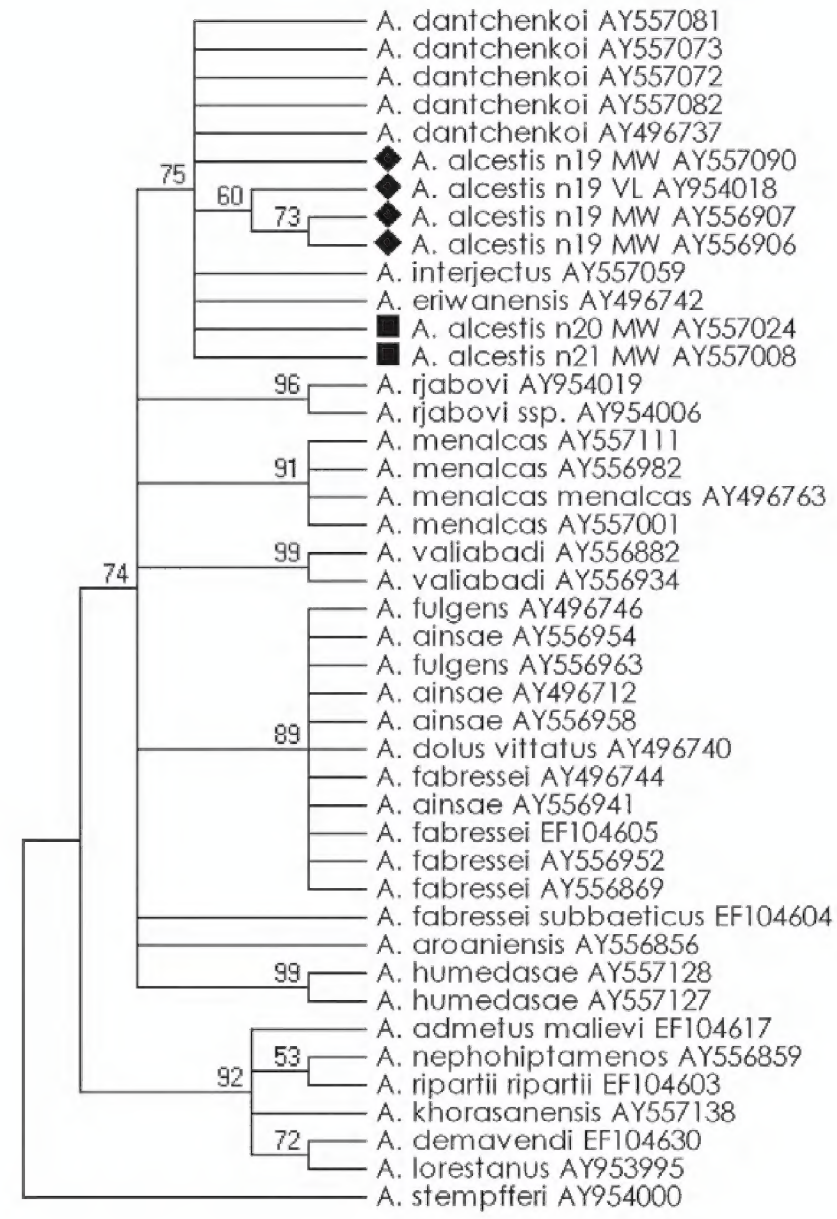

Fig. 3. Bootstrap consensus MP tree of the "brown" *Agrodiaetus* complex inferred from *COI* barcodes. Bootstrap values >50% are shown above the branches. Haploid chromosome number of *A. alcestis* are shown after name of a taxon.

Markazi provinces *A. alcestis karacetinae* and *A. demavendi* were sympatric in their distribution (Fig. 5). In all these localities imago of both species flow together: syntopically and synchronously. The stable chromosome number n=19 was found in all the studied populations of A. *alcestis karacetinae*. This chromosome number was also established in other populations from NW Iran (de Lesse, 1960b) and SE Turkey (Lukhtanov et al., 1998; Lukhtanov, Dantchenko, 2002a, 2002b), whereas *A. alcestis alcestis* from other parts of Turkey and from Lebanon had n=20 or n=21 (de Lesse, 1960b; Larsen, 1975). In populations of *A. demavendi* chromosome numbers were not stable: there was a tendency towards increasing the chromosome numbers from n=64-67 in the south of revealed distributional area (Esfahan and Lorestan provinces) to n=73-74 in the north (Kurdestan) (Fig. 5-6). Thus, despite the morphological similarity, *A. alcestis* sensu lato and *A. demavendi* can be easily distinguished by their karyotypes. Fereydun-Shahr (province Esfahan) was the southernmost locality where *A. alcestis karacetinae* and *A. demavendi* were discovered by us. This locality seems to be close to the southernmost limit of entire distribution ranges of these species, as no representatives

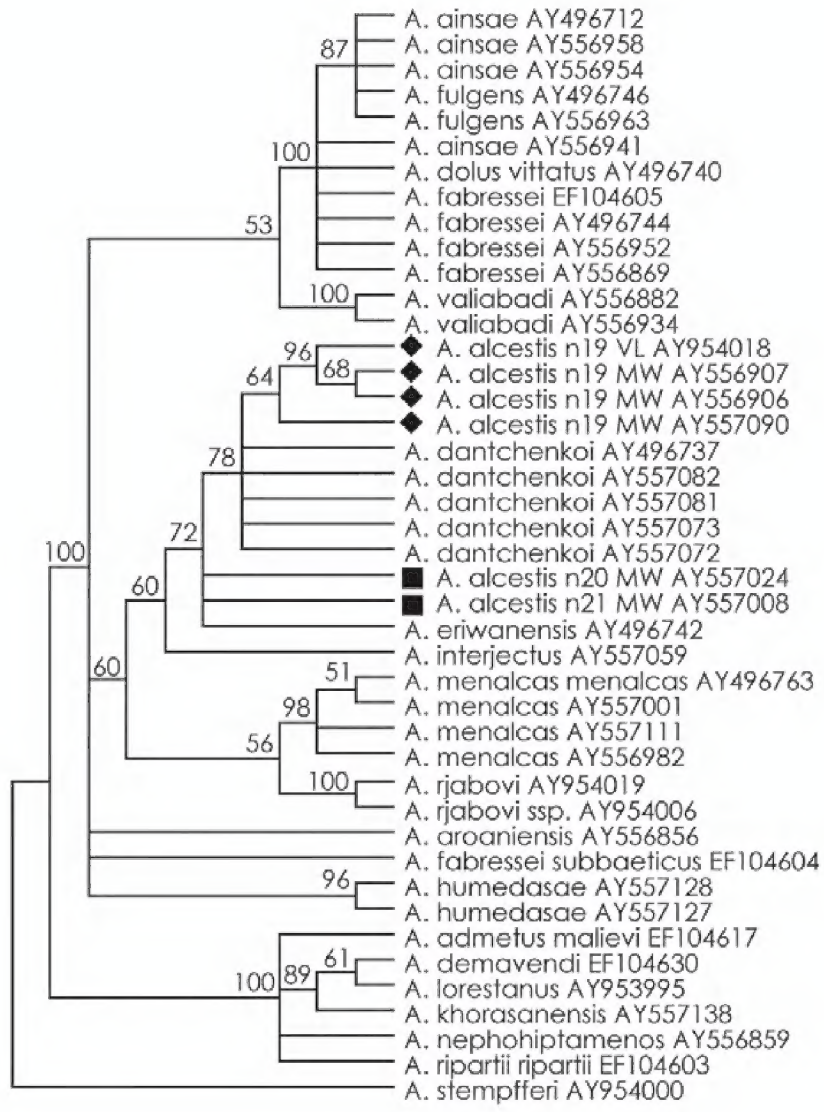

Fig. 4. Consensus Bayesian tree of the "brown" *Agrodiaetus* complex inferred from *COI* barcodes. Posterior probability values >50% are shown above the branches. Haploid chromosome number of *A. alcestis* are shown after name of a taxon.

of the "brown" complex are known from more southern regions (Nazari, 2003). At the same time, this locality seems to be close to the easternmost limit of distribution range of *A. alcestis* sensu lato (Fig. 6).

In Zanjan (West part), Azerbaijan-e-Gharbi and Ardebil provinces we found specimens with chromosome numbers n=77, n=80 (Fig. 1, c, Table 1). These chromosome numbers as well as the structure of entire karyotype are similar to those known in *A. admetus*, another representative of the "brown" complex. *A. admetus* is known from Balkan Peninsula, Turkey, Armenia, and Azerbaijan (Kandul et al., 2007). Although *A. admetus* was previously mentioned for Iran (see: Carbonell, 2001: 106), this record was not confirmed by chromosomal or molecular data. Thus, our finding seems to represent the first confirmed evidence for the presence of *A. admetus* in Iran.

In Azerbaijan-e-Sharqi province we found a specimen with chromosome number n=ca.89 (Fig. 1, d, Table 1). This chromosome number as well as the karyotype structure is similar to those known in *A. ripartii* (Freyer, 1830) (Lukhtanov, Dantchenko, 2002a, b). *A. ripartii* was not previously mentioned for Iran, except for *A. ripartii eriwanensis* Forster, 1960 (Nazari, 2003). However, the latter record was not confirmed by chromosomal or molecular data. It should be also noted that the taxon *A. eriwanensis* is not closely related to *A. ripartii* (see: Figs 2-4). Thus, our finding seems to represent the first evidence for the occurrence of *A. ripartii* in Iran.

Our karyological studies confirm the conclusion of de Lesse (1960a, 1960b), that species determination within the "brown" complex is impossible without investigation of karyotypes. Preliminary species determinations made by us in the field, were proved to be incorrect in many cases. We found two sorts of errors: (a) specimens recognized as *A. alcestis* turned out to be *A. demavendi* or vice versa, (b) specimen recognized as *A. alcestis* or *A. demavendi* turned out to be another species (*A. admetus*, *A. ripartii*).

Wiemers (2003) proposed a hypothesis about non-conspecificity of *A. alcestis karacetinae* and *A. alcestis alcestis*. We tested this hypothesis by analysing the Wiemers' original *COI* sequences as well as other samples from GenBank representing additional target and outgroup taxa. The analysis of more representative data set generally confirmed this hypothesis. It is demonstrated that *A. alcestis* sensu lato

Fig. 5. Distribution map of *A. alcestis karacetinae* (white circle) and *A. demavendi* (white square) in Iran with their haploid chromosome numbers (original data).

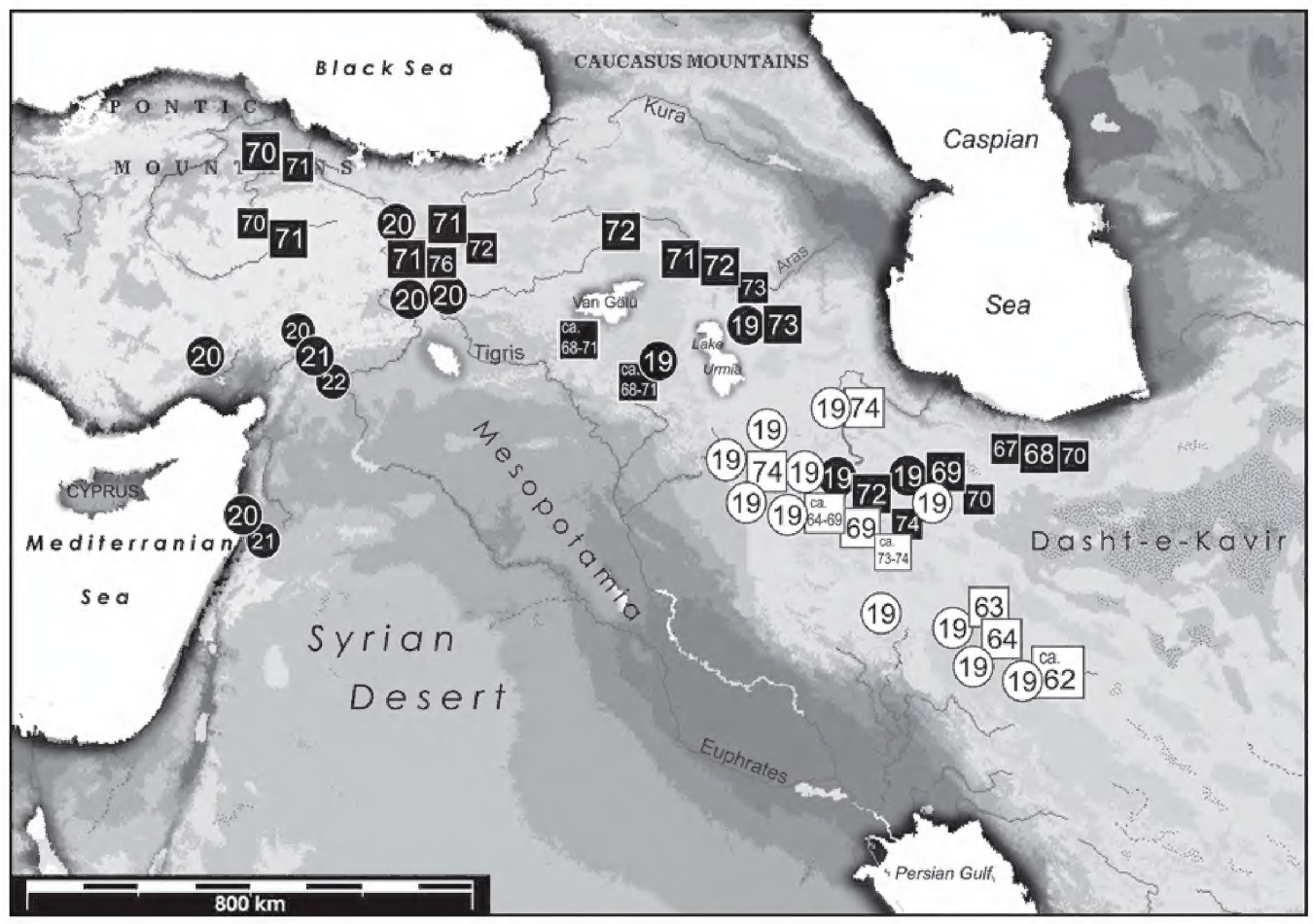

Fig. 6. Distribution map of *A. alcestis alcestis* (n=20, n=21), *A. alcestis karacetinae* (n=19) and *A. demavendi* (n=64-74). Original data are shown by white circles and squares, the data by de Lesse (1960b) and Larsen (1975) are shown by black circles and squares.

(*A.alcestis karacetinae* + *A. alcestis alcestis*) represents a polyphyletic taxon consisting of most likely not sister species: *A. karacetinae* (n=19) and *A. alcestis* (n=20, n= 21). However, we note that this conclusion can not be considered final. Since *A. dantchenkoi* and *A. karacetinae* are parapatric in distribution (Lukhtanov et al., 2003), we can not exclude that the similarity between these taxa in *COI* barcodes is a consequence of mitochondrial introgression between them and does not reflect their close relatedness. The possibility of interspecific mitochondrial introgression was recently demonstrated in Lepidoptera (Lukhtanov, Shapoval, 2008; Lukhtanov et al., 2009). Therefore, the conclusion about not-sister relationship between *A. karacetinae* and *A. alcestis* sensu stricto should be checked in the future studies by analyzing not only mitochondrial, but also nuclear molecular markers.

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